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Bactericidal Activities of Chloramphenicol and Eleven Other Antibiotics Against *Salmonella* spp.

STEPHEN R. PREBLUD,[1]†* CHARLES J. GILL,[2] AND JOSEPH M. CAMPOS[1,2]

Infectious Diseases Division[1] and Clinical Microbiology Laboratory,[2] The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104

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The bactericidal activity of chloramphenicol against 27 strains of *Salmonella typhi* and 33 strains of *S. enteritidis* was compared with those of 11 other antibiotics. The geometric mean bactericidal concentrations of chloramphenicol against susceptible strains (36.10 and 43.13 μg/ml for *S. typhi* and *S. enteritidis*, respectively) far exceeded those of the other 11 antibiotics, with cephalothin having the next highest values (2.67 and 8.66 μg/ml) and moxalactam (0.09 and 0.28 μg/ml), cefotaxime (0.08 and 0.28 μg/ml), ceftriaxone (0.07 and 0.16 μg/ml), norfloxacin (0.06 and 0.10 μg/ml), and aztreonam (0.05 and 0.20 μg/ml) having the lowest values. The results for imipenem (0.24 and 0.81 μg/ml) and ceftazidime (0.22 and 0.75 μg/ml) were lower than those noted for trimethoprim-sulfamethoxazole (1.20 and 5.56 μg/ml), cefamandole (0.62 and 3.29 μg/ml), and ampicillin (0.55 and 2.78 μg/ml). The MBC of chloramphenicol for some isolates decreased with increased incubation times such that the proportion of susceptible isolates killed by chloramphenicol at concentrations within achievable levels in blood increased from 10% after 24 h to 26% after 48 h of incubation. Although the MBCs of the other 11 antibiotics for some isolates were also lowered by prolonged incubation, all 24-h values were within achievable levels in blood. The data indicate that chloramphenicol is not uniformly bacteriostatic against *S. typhi* and *S. enteritidis*. The in vivo significance of demonstrating delayed killing by chloramphenicol is, however, uncertain.

Chloramphenicol is generally regarded as a bacteriostatic antibiotic for *Salmonella* spp. (19, 22). However, as with other members of the family *Enterobacteriaceae* (5, 12), there are occasional isolates that are killed in vitro with achievable chloramphenicol levels in blood. Recently, Asmar and Dajani (3) reported that chloramphenicol was bactericidal for 2 of 13 *Salmonella* isolates. To more accurately quantitate this property, we evaluated the bactericidal activity of chloramphenicol for 27 *S. typhi* and 33 *S. enteritidis* strains and compared the results with those obtained for 11 other antibiotics (ampicillin, trimethoprim-sulfamethoxazole [TMP-SMZ], cephalothin, cefamandole, moxalactam, cefotaxime, ceftriaxone, ceftazidime, imipenem, aztreonam, and norfloxacin). In addition, we studied the effect of incubation time on MIC and MBC results. Specifically, we arbitrarily used an MBC/MIC ratio of ≥10 for each antibiotic against susceptible strains as a relative measure of discrepant MBCs and MICs and hence as a relative indicator of bactericidal versus bacteriostatic activity. Values at 24 and 48 h were compared to investigate conditions that could alter this ratio and its prevalence among the *Salmonella* strains tested.

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MATERIALS AND METHODS

The *Salmonella* isolates were obtained from Frances Hickman at the Centers for Disease Control, from Bruce Kleger at the Pennsylvania State Department of Health, and from the clinical microbiology laboratory at The Children's Hospital of Philadelphia.

Agar disk diffusion susceptibility results were determined for each isolate (13). Broth dilution susceptibility studies were carried out in duplicate in microtiter trays with Mueller-Hinton broth (14). The antibiotic concentrations used ranged from 0.05 to 100 μg/ml. Each well was inoculated to a cell density of ca. 100,000 CFU/ml. Pairs of inoculation trays were incubated at 37°C, one tray for 24 h and one tray for 48 h. The MIC was defined as the lowest concentration of antibiotic able to inhibit visible growth of the test organism. The contents of each clear well (0.1 ml) were spotted onto the surface of a predried Mueller-Hinton agar plate. After the spots had soaked into the agar, the plate was incubated at 37°C for 24 h. The MBC was defined as the lowest concentration of antibiotic able to kill at least 99.9% of the original inoculum (i.e., ≤10 colonies per spot).

Summary MICs and MBCs were calculated including only susceptible strains. For calculation of the geometric mean (GM) MICs and MBCs, results that were equal to or less than the lowest concentration tested were used as is, whereas results that were greater than the highest concentration tested were arbitrarily assigned a value that was one dilution higher.

RESULTS

Based on both disk diffusion and MIC results, 6 (22%) of the 27 *S. typhi* strains were resistant to chloramphenicol. One of these six isolates was also resistant to ampicillin, whereas one other was also resistant to TMP-SMZ. The *S. enteritidis* isolates were more resistant. Of the 33 isolates, 17 (52%) were resistant to ampicillin. These 17 strains included 4 (12%) that were also resistant to chloramphenicol and 9 (27%) others that were also resistant to both cephalothin and cefamandole.

In general, MICs and MBCs for *S. enteritidis* were higher than those for *S. typhi* (Tables 1 and 2). Chloramphenicol and cephalothin had the highest GM MICs for both *S. typhi* and *S. enteritidis* (for chloramphenicol, 2.60 and 2.31 μg/ml, respectively; for cephalothin, 1.76 and 3.04 μg/ml, respectively) (Table 1). On the other hand, moxalactam, cefotax-

* Corresponding author.
† Present address: Division of Immunization, Center for Prevention Services, Centers for Disease Control, Atlanta, GA 30333.

TABLE 1. Comparative MICs of 12 antibiotics against susceptible[a] *Salmonella* isolates

Antibiotic	MIC (μg/ml)							
	GM		Range		For 50% of isolates		For 90% of isolates	
	S. typhi	*S. enteritidis*	*S. typhi*	*S. enteritidis*	*S. typhi*	*S. enteritidis*	*S. typhi*	*S. enteritidis*
Chloramphenicol	2.60	2.31	2.50–5.00	≤0.50–2.50	2.50	2.50	2.50	2.50
Cephalothin	1.76	3.04	1.00–5.00	0.10–10.00	2.50	2.50	2.50	10.00
TMP-SMZ	0.71	0.96	0.50–2.50	≤0.50–5.00	0.50	1.00	2.50	2.50
Cefamandole	0.41	1.07	0.25–2.50	0.10–10.00	0.50	1.00	1.00	5.00
Ampicillin	0.35	0.89	0.25–1.00	0.10–10.00	0.25	1.00	0.50	2.50
Imipenem	0.22	0.39	0.10–0.25	≤0.05–2.50	0.25	0.50	0.25	0.50
Ceftazidime	0.17	0.42	0.10–0.25	≤0.05–5.00	0.25	0.50	0.25	1.00
Moxalactam	0.06	0.08	≤0.05–0.10	≤0.05–0.50	0.25	0.10	0.10	0.10
Cefotaxime	0.06	0.10	≤0.05–0.10	≤0.05–0.25	≤0.05	0.10	0.10	0.10
Ceftriaxone	0.05	0.06	≤0.05–0.10	≤0.05–0.25	≤0.05	≤0.05	0.10	0.10
Norfloxacin	0.05	0.06	≤0.05–0.10	≤0.05–0.10	≤0.05	≤0.05	≤0.05	0.10
Aztreonam	0.05	0.08	≤0.05–0.10	≤0.05–0.25	≤0.05	0.10	≤0.05	0.10

[a] Excludes 6 chloramphenicol-, 1 TMP-SMZ-, and 1 ampicillin-resistant *S. typhi* isolate and 4 chloramphenicol-, 9 cephalothin-, 9 cefamandole-, and 17 ampicillin-resistant *S. enteritidis* isolates.

ime, ceftriaxone, norfloxacin, and aztreonam had the lowest GM MICs, ranging from 0.05 to 0.10 μg/ml.

The GM MBCs for chloramphenicol were 36.10 μg/ml for *S. typhi* and 43.13 μg/ml for *S. enteritidis*, both of which far exceeded the results for the 11 other antibiotics (Table 2). Of these, cephalothin had the highest values (2.67 μg/ml for *S. typhi* and 8.66 μg/ml for *S. enteritidis*), whereas moxalactam, cefotaxime, ceftriaxone, norfloxacin, and aztreonam again had the lowest values for all strains tested (0.05 to 0.28 μg/ml). As was the case for the GM MICs, the GM MBCs for imipenem and ceftazidime were lower than those noted for ampicillin, TMP-SMZ, and cefamandole.

The same relative differences among the antibiotics were noted for the MICs and the MBCs for 50 and 90% of the isolates (Tables 1 and 2). There were no significant differences in our observations when only the ampicillin- and chloramphenicol-resistant strains were included in the analysis.

Discrepancies between MBCs and MICs after 24 and 48 h of incubation are presented in Table 3. After 24 h, an MBC/MIC ratio of ≥10 occurred most frequently for chloramphenicol, involving 18 of 21 chloramphenicol-susceptible *S. typhi* strains and 27 of 29 chloramphenicol-susceptible *S. enteritidis* strains. Overall, for 45 (90%) of these 50 *Salmonella* strains, chloramphenicol MBCs (≥25 μg/ml) were at least 10 times the MICs. For the five uninvolved strains, chloramphenicol MBCs ranged from 0.50 to 10.0 μg/ml, which are within achievable chloramphenicol levels in blood.

After 48 h of incubation, chloramphenicol still had an MBC/MIC ratio of ≥10 against 75% of the *S. typhi* and 72% of the *S. enteritidis* strains (Table 3). For a total of eight strains, for which there previously were discrepant chloramphenicol MBCs at 24 h, there were low chloramphenicol MBC/MIC ratios at 48 h, with MBCs ranging from 2.50 to 10.0 μg/ml, all within achievable levels in blood. The overall proportion of *Salmonella* strains for which there were discrepant chloramphenicol MBCs decreased from 90% after 24 h to 74% after 48 h of incubation.

The change in the 24- and 48-h MBCs and MICs of chloramphenicol for the *S. typhi* and *S. enteritidis* strains initially with a ratio of ≥10 are presented in Fig. 1. The GM MBC decreased from 48.23 to 22.54 μg/ml for the *S. typhi* isolates and from 63.64 to 22.19 μg/ml for the *S. enteritidis* isolates. There was virtually no change in the GM MICs.

The other 11 antibiotics had discrepant MBCs after 24 h of incubation against relatively few of the *S. typhi* strains (Table 3). Only five antibiotics (TMP-SMZ, ampicillin, cefotaxime, ceftazidime, and norfloxacin) exhibited this phenomenon and did so at a rate no higher than 12% (TMP-

TABLE 2. Comparative MBCs of 12 antibiotics against susceptible[a] *Salmonella* isolates

Antibiotic	MBC (μg/ml)							
	GM		Range		For 50% of isolates		For 90% of isolates	
	S. typhi	*S. enteritidis*	*S. typhi*	*S. enteritidis*	*S. typhi*	*S. enteritidis*	*S. typhi*	*S. enteritidis*
Chloramphenicol	36.10	43.13	2.50–>100.00	0.50–>100.00	25.00	50.00	>100.00	>100.00
Cephalothin	2.67	8.66	1.00–10.00	0.10–25.00	2.50	10.00	5.00	25.00
TMP-SMZ	1.20	5.56	≤0.50–10.00	0.50–50.00	1.00	10.00	5.00	50.00
Cefamandole	0.62	3.29	0.25–2.50	0.10–25.00	0.50	5.00	2.50	25.00
Ampicillin	0.55	2.78	0.25–2.50	≤0.10–25.00	0.50	5.00	1.00	10.00
Imipenem	0.24	0.81	≤0.05–0.50	≤0.05–10.00	0.25	1.00	0.50	5.00
Ceftazidime	0.22	0.75	≤0.10–1.00	≤0.05–10.00	0.25	0.50	0.25	10.00
Moxalactam	0.09	0.28	≤0.05–0.25	≤0.05–2.50	≤0.05	0.25	0.25	1.00
Cefotaxime	0.08	0.28	≤0.05–0.50	≤0.05–1.00	0.10	0.25	0.25	1.00
Ceftriaxone	0.07	0.16	≤0.05–0.25	≤0.05–1.00	≤0.05	0.10	0.25	0.50
Norfloxacin	0.06	0.10	≤0.05–0.10	≤0.05–1.00	≤0.05	0.10	≤0.05	0.25
Aztreonam	0.05	0.20	≤0.05–0.25	≤0.05–2.50	≤0.05	0.10	≤0.05	1.00

[a] Excludes 6 chloramphenicol-, 1 TMP-SMZ-, and 1 ampicillin-resistant *S. typhi* isolate and 4 chloramphenicol-, 9 cephalothin-, 9 cefamandole-, and 17 ampicillin-resistant *S. enteritidis* isolates.

TABLE 3. Susceptible *Salmonella* strains with an MBC/MIC ratio of ≥10 at 24 and 48 h of incubation

Antibiotic	No.[a] (%)			
	S. typhi		*S. enteritidis*	
	24 h	48 h	24 h	48 h
Chloramphenicol	18/21 (86)	15/20 (75)[b]	27/29 (93)	21/29 (72)
TMP-SMZ	3/26 (12)	0/26 (0)	17/33 (52)	0/33 (0)
Moxalactam	0/27 (0)	0/27 (0)	11/33 (33)	0/33 (0)
Aztreonam	0/27 (0)	0/27 (0)	9/33 (27)	0/33 (0)
Cefamandole	0/27 (0)	0/27 (0)	8/24 (33)	0/24 (0)
Ceftazidime	1/27 (4)	0/27 (0)	8/33 (24)	0/33 (0)
Cefotaxime	1/27 (4)	0/27 (0)	7/33 (21)	0/33 (0)
Ceftriaxone	0/27 (0)	0/27 (0)	7/33 (21)	0/33 (0)
Imipenem	0/27 (0)	0/27 (0)	7/33 (21)	0/33 (0)
Ampicillin	1/26 (4)	0/27 (0)	4/16 (25)	0/16 (0)
Cephalothin	0/27 (0)	0/27 (0)	4/24 (17)	0/24 (0)
Norfloxacin	1/27 (4)	0/27 (0)	2/33 (6)	0/33 (0)

[a] Number of susceptible isolates with an MBC/MIC ratio of ≥10/ total number of susceptible isolates.
[b] MBC at 48 h not obtained for one isolate.

SMZ). On the other hand, all the other antibiotics had discrepant MBCs for the *S. enteritidis* strains, with rates varying from a high of 52% for TMP-SMZ to a low of 6% for norfloxacin. Furthermore, whereas inordinately high MBCs for more than one antibiotic besides chloramphenicol occurred with only 5% of the *S. typhi* strains, this property was noted with 41% of the *S. enteritidis* strains. In no case, however, did the MBCs of the other 11 antibiotics for any isolate exceed achievable levels in blood.

After 48 h of incubation, none of the other 11 antibiotics had an MBC/MIC ratio of ≥10 against any of the *Salmonella* strains (Table 3). The change in MBCs and MICs observed for TMP-SMZ against the *S. enteritidis* strains initially with an MBC/MIC ratio of ≥10 are depicted in Fig. 2. As was the case for chloramphenicol, the change in the MBC/MIC ratio was due to a change in MBCs. The 24- and 48-h GM MBCs were 17.42 and 1.25 μg/ml, respectively; the corresponding GM MICs were 0.78 and 1.07 μg/ml, respectively.

DISCUSSION

Our data document that *S. typhi* and *S. enteritidis* are exquisitely susceptible in vitro to a number of newly licensed and soon-to-be-licensed antibiotics. Although comparisons of antibiotic activities frequently include resistant organisms, we excluded such isolates to prevent an artificial increase in the difference between antibiotics to which resistance has occurred and those to which resistance has not yet been observed.

These findings are important in light of chloramphenicol and ampicillin (and amoxicillin) resistance. Unfortunately, in vitro results for *Salmonella* spp. may not correlate with clinical response (1, 7, 24). Although cefazolin may be effective (24), the only alternative antibiotic to date that has been relatively well studied and shown to be efficacious has been TMP-SMZ (4); however, resistance to this antibiotic has been reported (20, 21). Fortunately, favorable results for other, newer antibiotics are accumulating (2, 5, 10).

Our results also document that, in general, chloramphenicol is bacteriostatic against *Salmonella* strains in vitro. However, the percentage of isolates that was killed by chloramphenicol at concentrations <10 times the MIC was influenced by the time of incubation, with an increase from 10% at 24 h to 26% after 48 h of incubation. Although the MBC/MIC ratio of ≥10 was arbitrarily chosen, it did correlate with MBCs that would not be attainable using standard chloramphenicol dosages. Of interest is the finding that the MBC/MIC ratios of ≥10 obtained after 24 h of incubation for the other 11 antibiotics uniformly decreased to low values after 48 h of incubation. However, the 24-h MBCs were still within achievable levels in blood.

The bacteriostatic nature of chloramphenicol may in some way be involved with the therapeutic problems associated with chloramphenicol therapy of *S. typhi*, namely, relapse and a carrier state (4, 27). In addition, the bacteriostatic activity of chloramphenicol should be considered when treating other serious *Salmonella* infections, especially meningitis (5, 15, 18, 19). Furthermore, there are some data suggesting that combining chloramphenicol with ampicillin to treat serious *Salmonella* infections may be useful only if

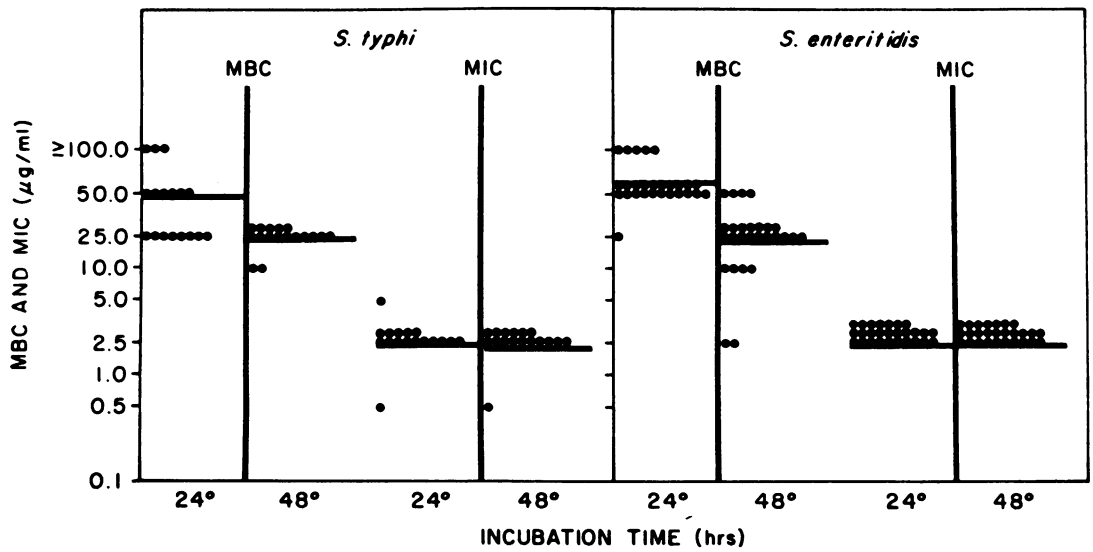

FIG. 1. Change in MBCs and MICs (for susceptible strains with an MBC/MIC ratio of ≥10 at 24 h of incubation) of chloramphenicol for *Salmonella* spp. with incubation time. ——, GM values.

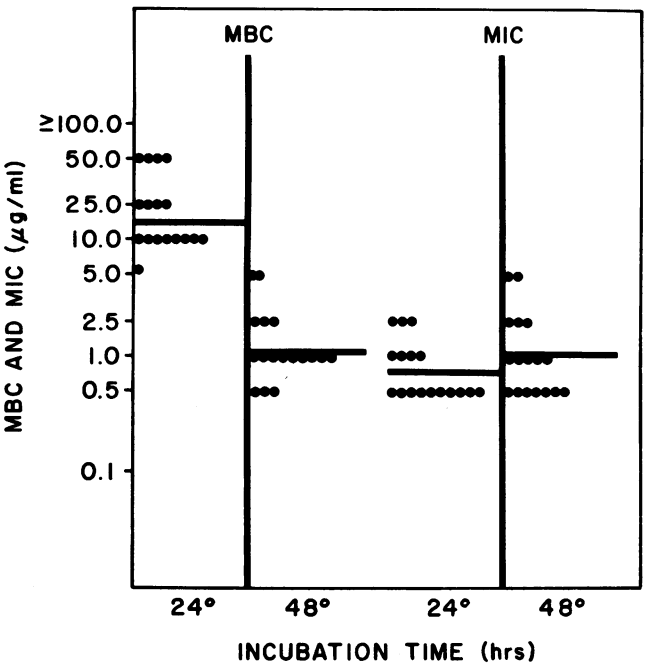

FIG. 2. Change in MBCs and MICs (for susceptible strains with an MBC/MIC ratio of ≥10 at 24 h of incubation) of TMP-SMZ for *S. enteritidis* spp. with incubation time. ——, GM values.

chloramphenicol is bactericidal for the particular organism (3). Otherwise, antagonism between the two antibiotics may occur. This finding may have clinical implications regarding routine combination therapy (3, 6, 8, 18).

Our observed laboratory-dependent changes in MBCs for the antibiotics studied are not unique. Alterations in MBCs have been documented for other antibiotics against other organisms after increased incubation times (23, 26) as well as after changes in broth (17), temperature (9), pH (25), and inoculum density (11, 16). Although the changes in MBCs noted after prolonged incubation may simply be in vitro phenomena, it is intriguing to speculate that similar changes do occur in the blood, cerebrospinal fluid, and tissues, assuming that it may take various times for susceptible organisms to be killed once they are exposed to a given antimicrobial agent. The true clinical importance of this laboratory phenomenon is, however, not known.

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LITERATURE CITED

1. **Adams, R., and J. D. Nelson.** 1968. Susceptibility of Salmonellae to cephalosporins and to nine other antimicrobial agents. Appl. Microbiol. **16**:1570–1574.
2. **Anton, P. A., J. A. Kemp, T. Butler, and M. R. Jacobs.** 1982. Comparative efficacies of ceftriaxone, moxalactam, and ampicillin in experimental *Salmonella typhimurium* infection. Antimicrob. Agents Chemother. **22**:312–315.
3. **Asmar, B. I., and A. S. Dajani.** 1983. Ampicillin-chloramphenicol interaction against enteric Gram-negative organisms. Pediatr. Infect. Dis. **2**:39–42.
4. **Butler, T., L. Rumans, and K. Arnold.** 1982. Response of typhoid fever caused by chloramphenicol-susceptible and chloramphenicol-resistant strains of *Salmonella typhi* to treatment with trimethoprim-sulfamethoxazole. Rev. Infect. Dis. **4**:551–561.
5. **Cherubin, C. E., M. L. Corrado, S. R. Nari, M. E. Gombert, S. Landesman, and G. Humbert.** 1982. Treatment of Gram-negative bacillary meningitis: role of the new cephalosporin antibiotics. Rev. Infect. Dis. **4**(Suppl.):S453–S463.
6. **Davis, R. C.** 1981. *Salmonella* sepsis in infancy. Am. J. Dis. Child. **135**:1096–1099.
7. **De Carvalho, E. M., R. Martinelli, M. M. G. De Oliveira, and H. Rocha.** 1982. Cefamandole treatment of salmonella bacteremia. Antimicrob. Agents Chemother. **21**:334–335.
8. **DeRitis, F., G. Giammanco, and G. Manzillo.** 1972. Chloramphenicol combined with ampicillin in treatment of typhoid. Br. Med. J. **4**:17–18.
9. **El Hagarawy, N., W. Lenz, A. Elkhoily, and A. H. El Molla.** 1982. The importance of incubation temperature for detecting beta-lactam-resistant *Staphylococcus aureus* strains. Infection **10**:371–374.
10. **Lé, C. T.** 1982. *Salmonella* vertebral osteomyelitis. Am. J. Dis. Child. **136**:722–724.
11. **Levin, R. M., P. H. Azimi, and M. G. Dunphy.** 1982. Susceptibility of *Haemophilus influenzae* type b to cefaclor and influence of inoculum size. Antimicrob. Agents Chemother. **22**:923–925.
12. **Meissner, H. C., and A. L. Smith.** 1979. The current status of chloramphenicol. Pediatrics **64**:348–356.
13. **National Committee for Clinical Laboratory Standards.** 1979. Performance standards for antimicrobial disc susceptibility tests. National Committee for Clinical Laboratory Standards, Villanova, Pa.
14. **National Committee for Clinical Laboratory Standards.** 1980. Standard methods for dilution antimicrobial susceptibility tests for bacteria which grow aerobically. National Committee for Clinical Laboratory Standards, Villanova, Pa.
15. **Nelson, S. J., and A. Granoff.** 1982. *Salmonella* gastroenteritis in the first three months of life: a review of management and complications. Clin. Pediatr. **21**:709–712.
16. **Neu, H. C.** 1978. Comparison of the pharmacokinetics of cefamandole and other cephalosporin compounds. J. Infect. Dis. **137**(Suppl.):S80–S87.
17. **Patamasucon, P., and G. H. McCracken, Jr.** 1982. Pharmacokinetics and bacteriological efficacy of *N*-formimidoyl thienamycin in experimental *Escherichia coli* meningitis. Antimicrob. Agents Chemother. **21**:390–392.
18. **Rabinowitz, S. G., and N. R. MacLeod.** 1972. *Salmonella* meningitis: a report of three cases and a review of the literature. Am. J. Dis. Child. **123**:259–262.
19. **Rahal, J. J., Jr., and M. S. Simberkoff.** 1979. Bactericidal and bacteriostatic action of chloramphenicol against meningeal pathogens. Antimicrob. Agents Chemother. **16**:13–18.
20. **Robins-Browne, R. M., B. Rowe, R. Ramsaroop, A. D. Naram, E. J. Threlfall, L. R. Ward, D. A. Lloyd, and R. E. Mickel.** 1983. A hospital outbreak of multiresistant *Salmonella typhimurium* belonging to phage type 193. J. Infect. Dis. **147**:210–216.
21. **Saad, A. F., and W. E. Farrar.** 1977. Antimicrobial resistance and R-factors in *Salmonella* isolated from humans and animals in Georgia and in South Carolina. South. Med. J. **70**:305–308.
22. **Smith, A. L., and A. Weber.** 1983. Pharmacology of chloramphenicol. Pediatr. Clin. North Am. **30**:209–236.
23. **Traczewski, M. M., D. A. Goldmann, and P. Murphy.** 1983. In vitro activity of rifampin in combination with oxacillin against *Staphylococcus aureus*. Antimicrob. Agents Chemother. **23**:571–576.
24. **Uwaydah, M.** 1976. Cefazolin in the treatment of acute enteric fever. Antimicrob. Agents Chemother. **10**:52–56.
25. **Venglarcik, J. S., III, L. L. Blair, and L. M. Dunkle.** 1983. pH-dependent oxacillin tolerance of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **23**:232–235.
26. **Winslow, D. L., J. Damme, and E. Dieckman.** 1983. Delayed bactericidal activity of β-lactam antibiotics against *Listeria monocytogenes*: antagonism of chloramphenicol and rifampin. Antimicrob. Agents Chemother. **23**:555–558.
27. **Woodward, T. E., J. E. Smadel, R. T. Parker, and C. L. Wisseman, Jr.** 1952. Treatment of typhoid fever with antibiotics. Ann. N.Y. Acad. Sci. **55**:1043–1055.